Chapter

# Hypoxic Preconditioning as a Strategy to Maintain the Regenerative Potential of Mesenchymal Stem Cells

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## Abstract

Mesenchymal stem cells (MSCs) are non-hematopoietic cells with high proliferative potential and multi-lineage differentiation capacity. MSCs are promising therapeutic candidates for cell-based therapies, and hundreds of clinical trials have been registered using these cells. Potential of stem cells is compromised with the factors such as disease condition and age of donor. Therefore, taking the cells from such patients for autologous use may compromise the benefits of cell-based therapies. It is therefore required to enhance the potential of these cells before use in stem cell-based therapies. Optimization of culture conditions is preferred strategies to enhance the regenerative potential of cells before use. This chapter briefly overviews the benefits of hypoxic preconditioning of stem cells to enhance the regenerative potential of cells in terms of their survival, proliferation, and differentiation.

**Keywords:** mesenchymal stem cells, hypoxic preconditioning, regenerative potentials

## 1. Introduction

Clinical use of stem cells is rapidly growing in recent years because of their capabilities to repair and regenerate tissues and organs of body [1]. Stem cells have self-renewal potential and can differentiate into cells of multiple lineages under appropriate conditions. They can secrete a large number of bioactive molecules that are involved in repair and regeneration of damaged tissues and organs. Based on their potential, stem cells are classified as totipotent, pluripotent, multipotent and unipotent. Fertilized eggs (zygote) are totipotent as they can make cells of all three lineages and extra embryonic tissue such as placenta. Embryonic stem cells (ESC) are derived from inner cell mass of 5 days old embryos and are pluripotent as they have potential to differentiate into all cells and tissues of the body except placenta. MSC are the most widely used cells and they can be isolated from various adult body tissues (bone marrow, adipose tissue, articular cartilage, synovium, synovial fluid, dental pulp, etc.) as well as from neonatal stem cell sources (cord blood, cord tissue, placenta) [2]. Currently, hundreds of clinical trials have been registered using MSCs (www.clinicaltrials.org) for various conditions such as degenerative brain disorders, stroke, cardiac dysfunctions, myocardial ischemia, renal disorders, wound healing, diabetes etc. [3]. MSCs exert their effect either by

transdifferentiation into respective tissues and/or through their paracrine effects by releasing different cytokines and growth factors [4].

The potential of adult stem cells such as MSCs is severely compromised in vitro by culture conditions and by number of passages of the cells [5]. In addition, "disease conditions" and "age" of the donor also reduces regenerative functionality of MSCs and their clinical use for repair and regeneration of damaged and lost tissues [6]. It is pertinent to note that elderly population is the main portion of population for potential stem cell-based regenerative therapies. However, autologous use of cells from aged individuals seems not to provide the expected benefits of stem cell-based therapies due to age depleted function of stem cells from such patients [7]. It is therefore required to enhance the potential of stem cells before clinical use. Different strategies have been employed for this purpose such as growth factors preconditioning [8], mild heat shock [9], and glucose depletion [10].

Different pretreatment strategies have been employed to enhance the regenerative potential of stem cells, however; hypoxic preconditioning seems more effective for enhancing stem cell function because relatively low oxygen concentrations prevail in stem cells niches as compared to normoxic conditions. Hypoxia can be an effective strategy for enhancing the cells function because it can make the cells adapt external microenvironment, reduce oxidative stress, shift metabolism towards glycolysis, enhance proliferation, differentiation and maintain stemness, and improve their motility to tolerate the hypoxic preconditioning after transplantation [11].

## 2. Regenerative potential of stem cells is compromised with age

### 2.1 Effect of donor age on regenerative potential of stem cells

Autologous stem cell-based therapies seem promising for several diseases. As humans get older, stem cell function deteriorate like other cells of the body. Diseases, especially degenerative diseases generally affect elderly people and therefore using autologous cells for such patients may have practical concerns [12]. With aging, superoxide dismutase activity (SOD) declines [13]. Studies indicate that differentiation potential of stem cells is negatively related with age of donor [12, 14–16], and therefore cell potential to form osteoblasts [12, 15–17], cartilage [12, 14–16] and other cell types is compromised. Another important aspect for cell-based therapies, that is, proliferation is also adversely effected with increasing donor age. Choudhery et al. [15] indicated that the number of population doublings decreased while the time of population doublings increased for cells obtained from aged donors as compared to cells from the young donors. Similarly, number of the colony forming units, size of colonies and plating efficiency of aged cells decreases in vitro [13–17]. Overall, growth kinetics and differentiation potential of the cells are inversely proportion to the donor age [15–18].

### 2.2 Effect of in vitro passaging

Cell-based therapies require large number of cells to get the favorable results in patients. For this purpose cells are expanded in vitro before use in most of clinical applications [12]. Leonard Hayflick in 1960 described that after a limited number of cell divisions the cells stop dividing. Cell morphology changes and they become enlarged and irregular in shape. The cells undergo a replicative senescence and this limited life span of cells is called as Hayflick's limit [19]. In this way replicative senescence limits the therapeutic potential of stem cells. The differentiation

potential of cells decreases with increasing number of in vitro passages. For example, bone marrow derived MSC showed decreased differentiation towards adipogenesis, osteogenesis and chondrogensis at late passages as compared to initial passages [20]. In addition, the proliferative potential of MSCs decreases after long term passages [21]. Human Wharton's jelly-derived mesenchymal stem cells showed significant decrease in growth kinetics and differentiation when cultured for longer time as compared to the cells in initial passages [22]. Feline adipose tissue derived MSC showed a progressive decrease in pluripotency and proliferation over continuous passaging [23].

## 3. Oxygen levels vary in tissues

The structural and functional microenvironment in tissues where stem cells reside is known as stem cell niche described for the first time by Schofield [24]. A cell niche maintains the identity and functional characteristics of resident cells [25]. Important identified stem cell niches are in bone marrow [26], vascular vessels [27], liver [28] adult kidneys [29] intestine [30] endometrium [31], oral tissue [32], skin [33] and adipose tissue [34]. **Table 1** shows variable oxygen concentrations in some important stem cell niches.

| Organ/tissues | PO2 values | References |
|---|---|---|
| Lungs (tracheal, bronchial, bronchiolar and alveolar epithelial cells) | 13–14% | [35] |
| Subcutaneous | 3–8% | [36] |
| Adipose tissue | 3–10% | [37, 38] |
| Heart | 2–6% | [39] |
| Brain (superficial cortex to deep white matter) | 3–5% | [40] |
| Brain (hypothalamus, hippocampus, midbrain) | 0.5% | [40] |
| Liver (parenchyma) | 4–7% | [41] |
| Kidney (renal cortex) | 4–9.5% | [42] |
| Kidney (medulla) | 2% | [42] |
| Pancreas (exocrine) | 2.7–4.6% | [43] |
| Pancreas (endogenous beta cell) | 5–6% | [43] |
| Stomach | 6–10% | [44] |
| Small intestine<br>Lumen<br>Mucosa<br>Serosa | 2–5%<br>3–6%<br>5–9% | [45] |
| Large intestine lumen and mucosa<br>Serosa | 0–2%<br>4–6% | [46] |
| Uterus | 2.5% | [47] |
| Bone marrow | 1–7% | [48] |
| Umbilical vein and arteries | 2.4–3.8% | [35] |
| Blood | 5–13% | [35] |

**Table 1.**
*Oxygen levels in different tissue in-vivo.*

It indicates that oxygen levels significantly vary in various tissues of the body and are significantly low as compared to normoxic oxygen concentration. Initially, oxygen level of 21% were adopted for in vitro culturing the cells based on normal oxygen conditions in environment, however, latter, it was realized that the cells grow better in vitro when cultured in those oxygen conditions which are representative of their respective niche.

Similarly, when cells are transplanted in the body, they face hypoxic in vivo environmental conditions [49, 50]. A large number of grafted cells die due to harsh in vivo environmental conditions (such as hypoxia) at transplanted site. The cell death due to hypoxic microenvironment is especially considerable for tissues that are not vascularized and or already injured or wounded [51].

## 4. How hypoxic preconditioning enhance stem cell function

### 4.1 Effect of hypoxia on gene transcription

The survival and functioning of stem cells in hypoxic environment depends upon their metabolic switch controlled by hypoxia inducible factors (HIFs). HIFs are transcription factors that are present in eukaryotes. They have two subunits, that is, alpha(α) and beta(β). Their α subunit has three isoforms (HIF1-3). The post translational modification of α subunit depends on hydroxylation which is oxygen dependent. When intracellular oxygen falls, α subunit forms a stable α/β dimer because hydroxylation did not occur. This dimer is transcriptionally active; it enters into the nucleus, binds to hypoxia response elements and initiates transcription of hypoxia sensitive genes [52, 53]. HIF3-α is the negative regulator of HIF1 and HIF2 (**Figure 1**).

Effect of hypoxia on HIF- α is different for different types of stem cells

GRP78-Akt axis induced by HIF1α is important in augmenting functions like proliferation and survival of MSCs under hypoxia [54].

### 4.2 Reactive oxygen species (ROS) and hypoxia

Reactive oxygen species are oxygen containing substances that are produced in cellular metabolism. They are detrimental to cellular functions. Hypoxic

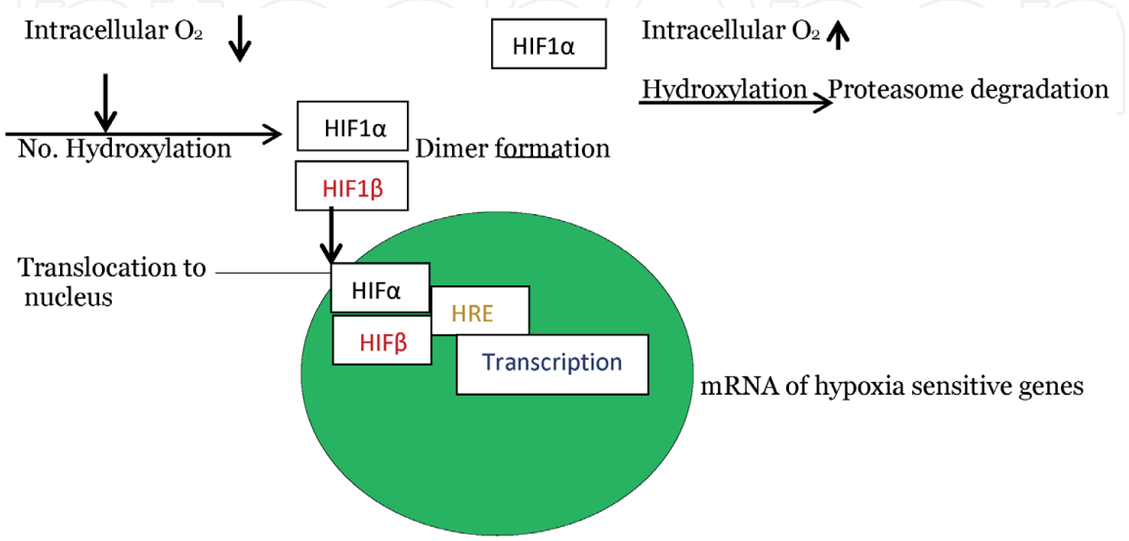


**Figure 1.**
*Mechanism of HIF1 formation and transcription of hypoxia sensitive genes. HIF (hypoxia inducible factor), HRE (hypoxia response element).*

preconditioning results in up regulation of Nuclear factor erythroid 2 related factor 2 (NRF2), which is a redox sensitive transcription factor involved in regulation of antioxidant genes [55]. Hypoxic preconditioning results in increased glycolytic metabolism and decreased tricarboxylic acid (TCA) cycle and oxidative phosphorylation. This mechanism leads to decreased mitochondrial ROS production and increase in the levels of antioxidant enzymes [52, 56]. Unbalanced redox homeostasis can cause stem cells aging and decreased proliferation. Hypoxic preconditioning augments redox metabolism [54] ROS acts paradoxically, at higher levels its cause's damage, and at lower levels it plays a role of signaling molecule. ROS also controls the hydroxylation of HIF1a, it causes inactivation of prolyl-hydroxylase enzymes (PHD), as a result degradation of alpha subunit does not occur and HIF1 formation occurs [57].

## 5. Hypoxic preconditioning improves regenerative potential of cells

### 5.1 Effect of hypoxia on stemness and survival of stem cells

Hypoxic preconditioning improves survival and stemness of cells and has been investigated in a number studies. SOX2, OCT4, NANOG and c-Myc are the markers that show stemness of cells. It has been found that stem cells grown in hypoxia are more viable, have decreased apoptosis through effects on HIF1a and p53 pathways [58].

When cultured under hypoxic (3 or 5% oxygen) condition for 5 days, stem cell markers were found to be statistically higher in dental pulp MSC [59].

PI3K/Akt signaling pathway get activated in cells exposed to hypoxia which in turn regulates many genes of cell cycle and CDK2 resulting in increased self-renewal and decreased apoptosis. Under hypoxic conditions cells switch their metabolism more towards glycolytic pathways and less towards oxidative phosphorylation resulting in less reactive oxygen species production (ROS) and more production of antioxidant enzymes [49, 60].

Hypoxia (1%) results in decreased senescence, increased lifespan of mesenchymal stem cells and were able to maintain proliferation rate, morphology and genetic stability [61]. Cryopreserved adipose derived stem cells cultured at 2 and 5% oxygen tension resulted in increased number and viability as compared to counterparts grown at 21% oxygen. In addition, all stemness related gene expression NANOG, SOX-2, REX-1, and OCT-4 were much higher in hypoxia group than in normoxia group. Another group demonstrated the upregulation of stemness related genes OCT4, SOX2 and NANOG in MSC grown in 3% oxygen culture conditions [62, 63]. Stemness of MSCs remains preserved in hypoxic cultures. Hypoxia results in decreased expression of apoptotic BCL-2 and CASP3 and increased expression of anti-apoptotic genes [64]. Stem cells derived from apical papilla of wisdom teeth also showed increased proliferation and upregulation of SSEA4 which is an embryonic stem cell marker surface antigen. Human umbilical cord derived MSC grown in a culture under 5% oxygen showed better proliferation and maintenance of stemness [65]. MSC derived from different sources showed increased expression of pluripotency markers Oct4, C-Myc and Nanog when cultured at 5% oxygen concentration levels [66]. Low oxygen tension also helped iPSCs from liver cells to preserve their stemness and decrease the time to switch from G1 phase to S phase, increase proliferation but the physiological oxygen level of tissue of origin should be kept in mind because these cells were grown in 10% oxygen level. The same group showed that culturing these cells at very low level result in loss of stemness [67].

### 5.2 Hypoxia improves differentiation potential of cells

Most of the studies use stem cells for regenerative purposes. MSC are multipotent cells that can differentiate into adipocytes, osteoblasts, chondrocytes and neurons under appropriate conditions [68]. The main advantage of stem cell therapy is living biological replacement rather than palliation through drugs. The use of stem cells to replace functional loss of specific tissue is determined by effective differentiation [69].

The results of differentiation are controversial; this variability may be due to:

1. Use of stem cells from different sources [70].

2. Due to heterogenous population of cells with similar morphology [71].

3. Designing of delivery system for successful transplantation of stem cells [72].

Biomaterials can be designed to act as carriers for the local delivery of stem cells, support cells or molecular niche cues [73]. Basal nutrients, cell density, spatial organization, mechanical forces, growth factors and cytokines have a profound influence on hMSC differentiation [71]. The role of hypoxia preconditioning in differentiation of stem cells into other lineages seems controversial due to inconsistent results of various studies [74]. Different studies have used different hypoxia percentages with variable times. Previously, cells were usually insulted with hypoxia using hydrogen peroxide, however, new trigas incubators have been developed recently that creates the precise hypoxic conditions even for longer period of times [59].

It has been shown that placenta derived MSC showed up regulation of osteogenic genes including osteopontin (OPN), osteocalcin (OCN), and alkaline phosphatase (ALP) as well as increased mineralization at 5% oxygen levels [75]. Another study found an increased osteogenic differentiation of human MSCs cultured at 5% oxygen for 5 days [66]. Contrary to this, multilineage differentiation potential including osteogenic differentiation of tendon derived MSc was compromised in hypoxia cultures [76]. Minsheng Yang and colleagues found that 9% hypoxia increased osteogenesis whereas 1% results in decreased osteogenic potentials due to upregulation of Notch 1 expression [77]. Cells cultured throughout in hypoxic culture (5%) showed less osteogenic potential, less mineralization as compared to cells primed with hypoxia (5% for 7 days). These results also emphasis that appropriate time for hypoxia is important to maneuver the different potentials [78].

Gale et al. analyzed the chondrogenic differentiation potential of equine synovial membrane and bone marrow derived MSCs and found no appreciable difference between cells cultured either at 5% oxygen or in normoxic conditions for 28 days. The results of expression of chondrogenic genes SOX9, ACAN, and COL2b were also variable between the groups [79]. Similar to this Li J and Pei M found no significant differences in chondrogenic index between normoxic and 5% hypoxic culturing for 7 days in synovium derived MSCs [80]. On the contrary adipose derived MSC showed better chondrogenesis and upregulation of several chondrogenic specific genes when grown in 2% oxygen cultures [81]. Bae et al. found increased expression of COL2A1, ACAN, and the transcription factor SOX9 in synovium derived MSC cultured at 5% oxygen levels. They also observed increased proteoglycan, glycosaminoglycans and collagen II contents from pellets in hypoxic condition [82]. Henrionnet et al. observed the effects of 5% oxygen conditions on bone marrow derived MSC cultures for their chongrogenic potential and resulted

in efficient and strong overexpression of chondrogenic genes COL2A1, ACAN, SOX9, and COMP along with down regulation of osteogenic genes ALP, and RUNX2 [83].

Valorani et al. found that pre-exposure hypoxia of 2% oxygen level results in increased expression of adipogenic genes including peroxisome proliferator activated receptor γ (Pparγ), lipoprotein lipase (Lpl) and fatty acid binding protein 4 (Fabp4) and adipogenesis in MSC derived from murine adipose tissue [84]. Another research found enhanced adipogenesis of human adipose tissue mesenchymal stem-cell (hAT-MSC) exposed to 2% hypoxic conditions for 7 days before shifting to normoxia during differentiation [85]. 2% hypoxia resulted in increased adipogenic differentiation of dental pulp and periodontal ligament derived stem cells [86]. Choi JR et al. found decreased adipogenesis and decreased expression of adipogenic genes including LPL, PPARc and FABP4 under hypoxia (2%) as compared to normoxia cultures [87]. Another research also found a decreased differentiation of stem cells under hypoxia [88].

### 5.3 Effect of hypoxia on proliferative potential of cells

Hypoxic preconditioning results in enhanced proliferation and increased colony forming units as compared to mesenchymal stem cells cultured in normoxia. Higher oxygen tensions increase oxidative stress to cells and activate apoptosis [55]. Zhang et al. explored the effects of 1 and 5% oxygen culture conditions on rat bone marrow derived MSC and compared them with their counter parts at 18% oxygen level cultures. They found significant increase in proliferation along with upregulation of BCL2 (antiapoptotic gene) and down regulation of BAX (apoptotic gene) [89]. About 5% oxygen tension resulted in greater size, cell number and cell density of MSC colonies [90]. Antebi et al. evaluated the potential of cells at different oxygen concentrations and found that proliferative potentials of porcine MSC was higher at 1, 2, and 5% oxygen tensions as compared to normoxic conditions. They also found that 48 hour hypoxia in their study resulted in more proliferation as compared to proliferative potential of cells when cultured for longer times (10 days) [64]. Elabd et al. suggested that hypoxic preconditioning should be used as a strategy for in vitro expansion of MSC before their clinical use. They cultured human bone marrow MSCs in 5 and 20% oxygen and observed greater effects of hypoxia not on the regenerative potentials but also on the gene expressions of hypoxia exposed MSCs [91]. Notch2-c-Myc signaling cause's proliferation under hypoxia and inhibits apoptosis. Hypoxia can have a great effect on proliferation of MSCs [92]. Hypoxia (1%) increases the proliferation not only in early passages but also in late passages as compared to normoxic cultures and extends the lifespan of MSCs [61]. Significantly higher number of cells as well as increased viability of ADSC occurs in hypoxic conditions [93]. Rat bone marrow MSC cultured at 5% oxygen levels exhibited increased number of colonies and shorter population doubling time as compared to normoxic cultured cells [94]. Asadpoor Dezaki et al. showed increased expansion, population doublings, viability and colony forming unit fibroblasts in a group cultured in 2.5% oxygen tension than normoxia group [95].

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## Acronyms and abbreviation

| | |
|---|---|
| MSCs | mesenchymal stem cells |
| ESC | embryonic stem cells |
| iPSC | induced pluripotent stem cells |
| SOD | superoxide dismutase activity |
| HIFs | hypoxia inducible factors |
| HRE | hypoxia response element |
| ROS | reactive oxygen species |
| PHD | prolyl-hydroxylase enzymes |
| OPN | osteopontin |
| OCN | osteocalcin |
| ALP | alkaline phosphatase |
| Pparγ | peroxisome proliferator activated receptor γ |
| Lpl | lipoprotein lipase |
| Fabp4 | fatty acid binding protein 4 |
| NRF2 | nuclear factor erythroid 2 related factor 2 |


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